Immunopharmacology and Immunotoxicology, 2010, 1–7, Early Online

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BRIEF COMMUNICATIONS

p38-Targeted inhibition of interleukin-12 expression by ethanol extract from *Cordyceps bassiana* in lipopolysaccharide-activated macrophages

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Abstract

Cordyceps species have been known as ethnopharmacologically valuable mushroom in Korea, China, and Japan. This plant has been reported to exhibit a variety of pharmacological activities such as antioxidative, anticancer, anti-inflammatory, antidiabetic, and antiobesity effects. Although numerous pharmacological potentials of *Cordyceps* spp. have been demonstrated, immunomodulatory effect of *Cordyceps bassiana* has not been published yet. To evaluate its immunomodulatory activity, macrophages activated by lipopolysaccharide (LPS) were employed and the production of interleukin-12 (IL-12) was explored in terms of understanding its molecular inhibitory mechanism. Seventy percent of ethanol extract from *Cordyceps bassiana* (Cb-EE) was able to suppress the expression of IL-12, a cytokine regulating interferon-γ (IFN-γ)-producing T helper type 1 (Th1) polarization response, at the transcriptional levels. The inhibitory effect of Cb-EE seemed to be due to activator protein-1 (AP-1) translocation inhibition, according to immunoblotting analysis with nuclear fraction and luciferase assay. In agreement with this, Cb-EE strongly suppressed the phosphorylation of p38, a prime signal to stimulate AP-1 translocation and IL-12 production, strongly suppressed by SB203580, a p38 inhibitor. Furthermore, this extract also suppressed IFN-γ production in both phytohemaglutinin A and LPS-activated splenocytes. Our results suggest that Cb-EE can be applied as a Th1 response regulatory herbal medicine.

Keywords: Cordyceps bassiana; *interleukin-12; macrophage; AP-1 translocation; p38 activation*

Introduction

Interleukin-12 (IL-12) is one of the many important cytokines involved in the modulation of T-cell differentiation. This cytokine is mostly produced from antigen presenting cells (APCs) such as dendritic cells and macrophages after initial recognition, uptake, processing and presentation of exogenous antigens. The released IL-12 stimulates native T cells to differentiate into interferon-γ (IFN-γ)-secreting helper type I CD4+ T (Th1) cells, which modulate the production of IgG1 from B cells, upregulation of cytotoxic T lymphocyte response, and enhancement of natural killer cell activity. IL-12 is therefore known to play a critical role in regulating anticancer and antiviral responses. However, over-production of this cytokine is also considered as a serious immunopathological event found commonly in chronic atopic dermatitis and eczema,[(1)] indicating that controlling IL-12 production can be a potential therapeutic target for curing atopic dermatitis.[(2)]

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(Received 10 February 2010; revised 23 March 2010; accepted 29 March 2010)

ISSN 0892-3973 print/ISSN 1532-2513 online © 2010 Informa UK Ltd
DOI: 10.3109/08923973.2010.482137

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The production of IL-12 in APC is critically managed by the transcriptional activation. Of all the transcription factors involved redox-sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) are activated by the generated radicals.[3] The upregulation of these transcription factors requires for the formation of total signaling complex including various protein kinases [Src, Syk, phosphoinositide 3-kinase (PI3K), and Akt (protein kinase B)] for NF-κB translocation, and mitogen-activated protein kinases (MAPKs) [such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38] for AP-1 translocation.[4,5] Because excessive production of IL-12 plays a major role in the etiology of various chronic immunological disease, development of promising anti-IL-12 drugs targeted to upstream the signaling cascade should be valuable in terms of prevention or therapy of these diseases.[6]

Cordyceps species have been known as an ethopharmacologically valuable mushroom in Korea, China, and Japan. Representatives of this species are *Cordyceps sinensis* and *Cordyceps militaris*. These have been used as a tonic for longevity, endurance, and vitality.[7] In addition, these mushrooms have also been reported to display numerous pharmacological activities such as antioxidative, antiviral, anticancer, antifibrotic, anti-inflammatory, antinociceptive, antiangiogenic, anti-diabetic, and antiobesity effects.[7,8] Although a variety of biological activities of *Cordyceps* species has been explored, no reports on the biological effects of *Cordyceps bassiana* have been published till date. Therefore, in this study, we investigated the effect of 70% EtOH extract of *Cordyceps bassiana* (Cb-EE) on the modulation of Lipopolysaccharide (LPS)-induced IL-12 expression in macrophages.

Materials and methods

Materials

LPS, phytohemaglutinin A (PHA), tumor necrosis factor-α (TNF-α), forskolin, and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical (St Louis, MO). SB203580 was from Calbiochem (La Jolla, CA). Seventy percent of EtOH extract from *Cordyceps bassiana* was prepared by conventional extraction methods.[9] *Cordyceps bassiana* was identified by Prof. Jae Mo Sung (Kangwon National University, Chuncheon, Korea). A voucher specimen of this (number 278-Cb-1) was deposited in the herbarium of our laboratory. RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All other chemicals were of reagent grade. Antiphospho or total antibodies to p38, p65 (NF-κB), c-Jun, c-fos, β-actin, and lamin A/C were from Cell Signaling (Beverly, MA).

Cell culture

RAW264.7 and HEK293 cells were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), glutamine and antibiotics (penicillin and streptomycin) at 37°C with 5% CO2.

IFN-γ production

Splenocytes were prepared from the spleen of BALB/C mice killed by cervical dislocation under sterile conditions, using the method described previously.[10] Splenocytes (5×10^{-6} cells/mL) were incubated with T cell or B cell mitogens [PHA (10 μg/mL) or LPS (10 μg/mL)] in the presence of Cb-EE for 48 h.[11] Supernatants were assayed for IFN-γ content using IFN-γ ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK).

MTT assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously.[11]

Luciferase reporter gene activity assay

HEK293 cells (1×10^{-6} cells/mL) were co-transfected with one or two of plasmid constructs containing NF-κB-Luc, AP-1-Luc or cAMP response element binding (CREB)-Luc as well as MyD88 and β-galactosidase using the calcium phosphate method in a 12-well plate, as reported previously.[12] The cells were then further incubated in the absence or presence of TNF-α (20 ng/mL), PMA (0.1 μM) or forskolin (25 μM) and finally harvested for experiments 48 h after transfection. Luciferase assays were performed using the Luciferase Assay System (Promega, Madison WI).[13]

Extraction of total RNA and semiquantitative reverse transcriptase (RT)-PCR amplification

The total RNA from the LPS treated-RAW264.7 cells was prepared by adding TRIzol Reagent (Gibco BRL), as per the manufacturer's protocol. Semiquantitative RT reactions were conducted using MuLV RT as per the method reported previously.[14] The primers (Bioneer, Daejeon, Korea) for IL-12 and glyceraldehyde-3-phosphate dehydrogenase detection were used as previously reported.[15]

Preparation of cell lysate and immunoblotting

For total protein extraction: RAW264.7 cells were harvested, washed with cold PBS and lysed in lysis buffer

(20 mM TRIS-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin, 1 mM benzimidine and 2 mM phenylmethane sulphonylfluoride) for 30 min rotating at 4°C. Lysates were clarified by centrifugation at 16,000g for 10 min at 4°C. For nuclear protein extraction: Nuclear proteins were obtained by three steps. After the treatment, cells were harvested and lysed in 500 μL of lysis buffer (50 mM KCl, 0.5% Nonidet p40, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 20 μg/mL aprotinin, and 100 μM 1,4-dithiothreitol) on ice for 4 min. Cells lysates were centrifuged at 14,000 rpm for 1 min at 4°C. In the second step, the pellet was washed with the wash buffer, which was the same as the lysis buffer excluding Nonidet p40. In the final step, the nuclei were incubated with an extraction buffer (500 mM KCl, 10% glycerol, 10 mM HEPES, 300 mM NaCl, 0.1 mM 1,4-dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin) and centrifuged at 19,000g for 5 min. Supernatant was collected as nuclear protein extract. Soluble cell lysates were immunoblotted and phospho-ERK levels were visualized as previously reported.[16]

Statistic analysis

The Student's *t*-test and one-way analysis of variance were used to determine the statistical significance between values of the various experimental and control groups. *P* values of 0.05 or less were considered to be statistically significant.

Results and discussion

In the present investigation, Cb-EE strongly suppressed the expression of IL-12 p40 in LPS-activated RAW264.7 cells (Figure 1B), without altering normal cell viability (Figure 1A), indicating that Cb-EE could specifically block the transcriptional activation in response to LPS. To investigate the inhibitory mechanism of Cb-EE, we first explored the transcription factors involved in the expression of IL-12 in LPS-activated RAW264.7 cells. Reporter gene assay performed by transfection of DNA constructs with binding sequences of NF-κB, AP-1, and CREB was employed. As shown in Figure 2, Cb-EE down-regulated luciferase activity was induced by the activation of both AP-1 and CREB, but not NF-κB, in PMA-or TNF-α-treated HEK293 cells, suggesting that these transcription factors can be considered as the inhibitory targets of Cb-EE-mediated IL-12 expression. Because stimulation conditions by PMA or TNF-α in HEK293 cells is quite distinct from LPS-induced stimulation in RAW264.7 cells, the alternative to mimic toll-like receptor 4-mediated NF-κB, CREB, or AP-1 activation was employed in the present study. It has been found that overexpression of MyD88, an important adaptor protein mediating TLR signaling, can trigger NF-κB activation which was assessed by measuring luciferase activity.[17,18] We found that co-transfection with MyD88 and NF-κB dramatically enhanced luciferase activity up to 140-folds than normal (Figure 2A). In addition, MyD88 upregulated AP-1 or CREB-mediated luciferase activity up to 30–40% (Figure 2B right panel and Figure 2C right panel). Similarly, under MyD88 co-transfection conditions, Cb-EE did not suppress NF-κB-mediated luciferase activity, whereas AP-1- and CREB-mediated luciferase activity was significantly decreased by this extract (Figure 2B right panel and Figure 2C right panel), indicating that a signal(s), important for both AP-1 and CREB activation, is modulated by MyD88 but not involved in NF-κB activation that might be an immunopharmacological target in Cb-EE-mediated inhibition.

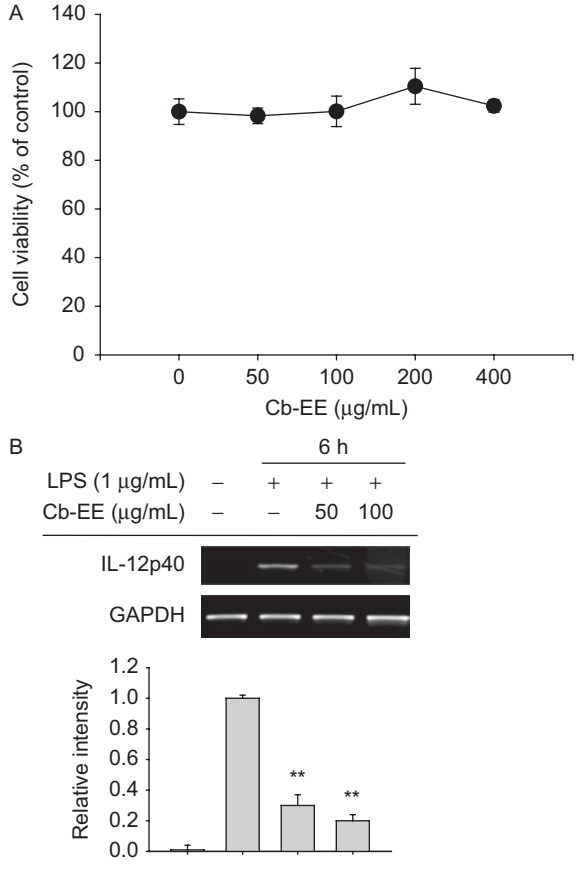

Figure 1. Effect of Cb-EE on the mRNA expression of interleukin-12 (IL-12) in lipopolysaccharide (LPS)-activated RAW264.7 cells. (A) The viability of RAW264.7 cells was determined by MTT assay. **P < 0.01 represents significant difference compared to control group. (B) RAW264.7 cells (2×10^{-6} cells/mL) were incubated with Cb-EE in the presence of LPS (1 μg/mL) for 6 h. The mRNA levels of IL-12 and glyceraldehyde-3-phosphate dehydrogenase were determined by RT-PCR.

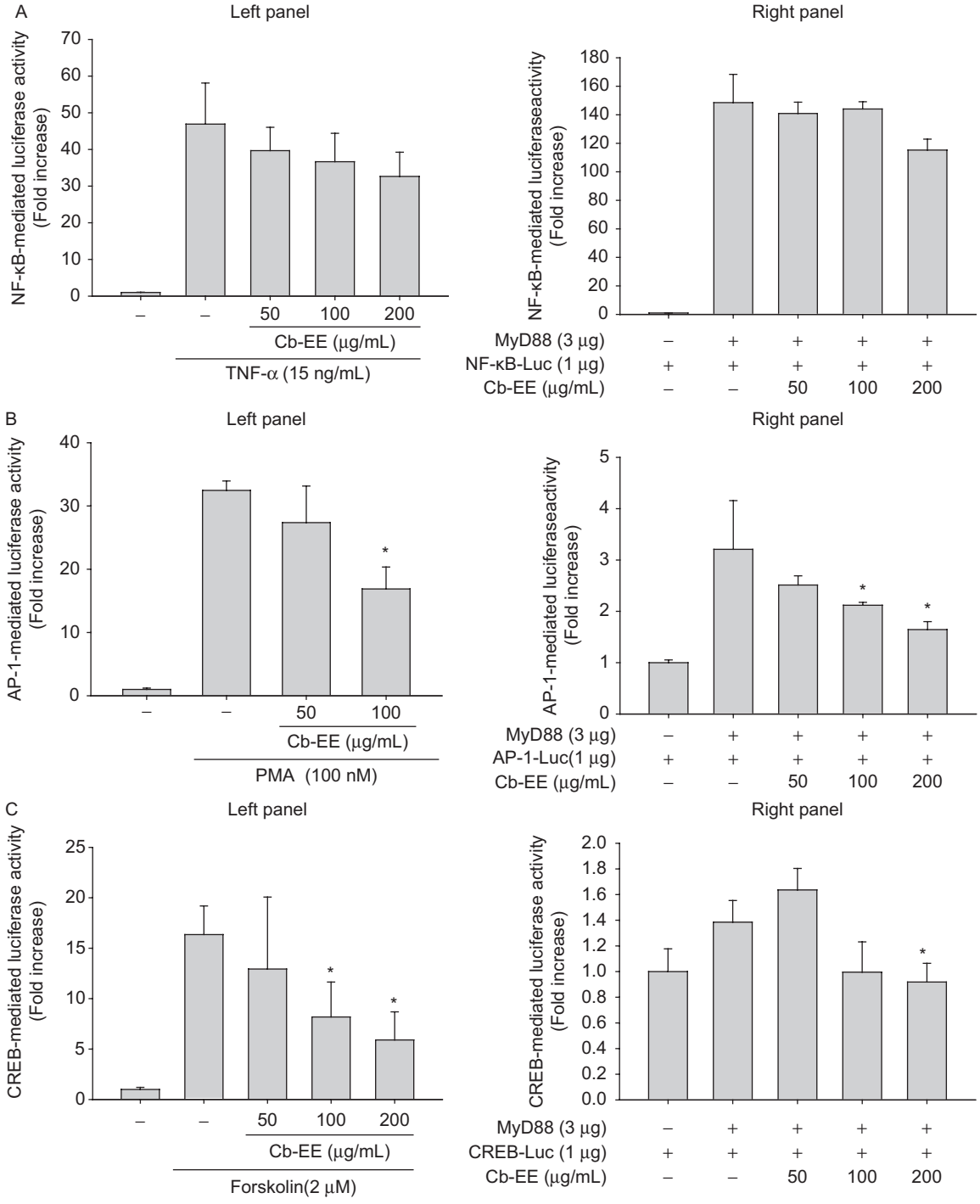

Figure 2. Effect of Cb-EE on the luciferase activity mediated by nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and cAMP response element binding (CREB). (A, B, and C) HEK293 cells co-transfected with one or two of plasmid constructs, MyD88, NF-κB-Luc, AP-1-Luc, or CREB-Luc (each 1 μg/mL) and β-gal (as a transfection control) were treated with Cb-EE in the presence or absence of PMA (100 nM), TNF-α (10 ng/mL) or forskolin (2 μM) for 12 h. Luciferase activity was determined by luminometry. *P < 0.05 represents significant difference compared to control group.

To confirm this inhibitory pattern, nuclear-translocated levels of transcription factors during Cb-EE treatment under LPS activation were determined by immunoblotting analysis. Figure 3 shows that the nuclear translocation of AP-1 [c-Jun (Figure 3B) and c-fos (Figure 3C)][19] but not NF-κB [p65 (Figure 3A)][20] triggered by LPS in RAW264.7 cells was blocked by Cb-EE exposure. However, in the case of CREB, there was no change of nuclear level during LPS treatment (Figure 3D). Instead, Cb-EE remarkably diminished upregulated level

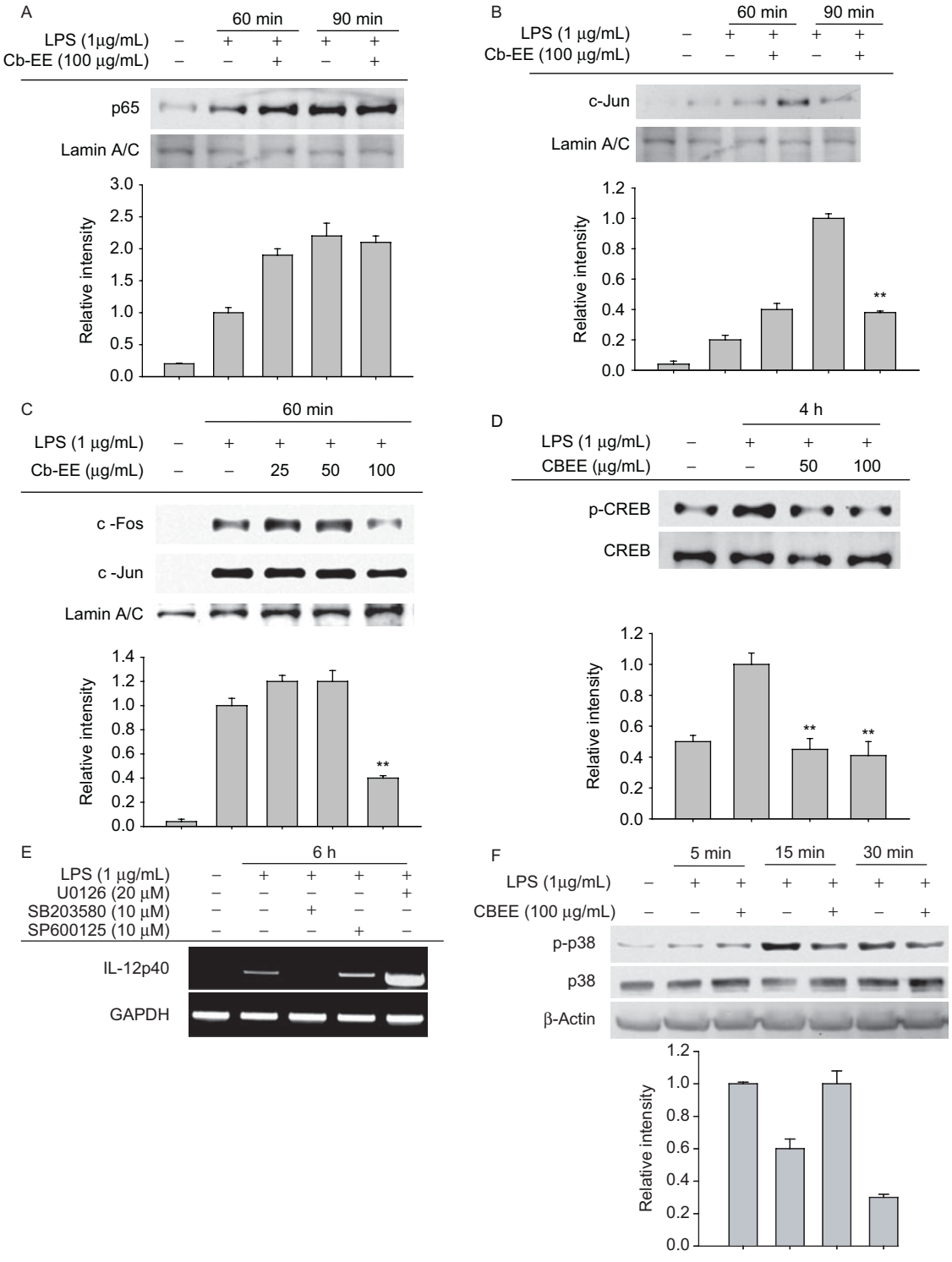

Figure 3. Effect of Cb-EE on the nuclear translocation of nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and cAMP response element binding (CREB), and their upstream signaling events. (A, B, C, and D) RAW264.7 cells (5×10^{-6} cells/mL) pretreated with Cb-EE for 1 h were stimulated in the absence or presence of lipopolysaccharide (LPS) (1 μg/mL) for indicated times. After preparation of nuclear fraction, the protein levels of p65, c-Jun, c-fos, and lamin A/C were determined by immunoblotting analysis with their total protein antibodies. (E) RAW264.7 cells (5×10^{-6} cells/mL) were incubated with U0126, SB203580, and SP600125 in the presence of LPS (1 μg/mL) for 6 h. The mRNA levels of IL-12 and glyceraldehyde-3-phosphate dehydrogenase were determined by RT-PCR. (F) RAW264.7 cells (5×10^{-6} cells/mL) pretreated with Cb-EE (300 μg/mL) were stimulated with LPS (1 μg/mL) for 30 min. After immunoblotting, the levels of phospho- or total p38 and β-actin were identified by their antibodies. **$P < 0.01$ represents significant difference compared to control group.

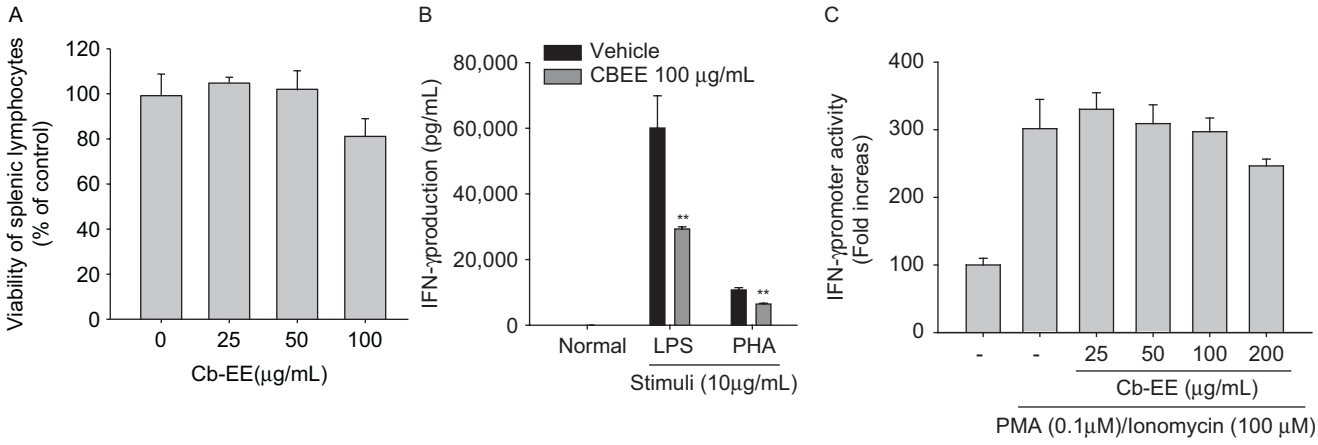

Figure 4. Effect of Cb-EE on the production of IFN-γ in lipopolysaccharide (LPS)- and phytohemaglutinin A (PHA)-treated splenocytes. (A) The viability of splenocytes (2×10^{-6} cells/mL) pretreated with Cb-EE was determined by MTT assay. (B) Splenocytes were incubated with Cb-EE in the presence of PHA (10 μg/mL) for 48 h. IFN-γ level in culture supernatant was determined by ELISA. (C) HEK293 cells co-transfected with IFN-γ-promoter-Luc (1 μg/mL) and β-gal (as a transfection control) were treated with Cb-EE in the presence or absence of PMA (100 nM) and ionomycin A (100 μM) for 12 h. Luciferase activity was determined by luminometry. **$P < 0.01$ represents significant difference compared to control group.

of phospho-CREB seen in LPS exposure (Figure 3D). Because it has been reported that CREB phosphorylation and AP-1 translocation can be managed by MAPK such as ERK, p38, and JNK,[21] we next evaluated the involvement of these enzymes in LPS-induced IL-12 gene expression in the macrophages by testing modulatory effects of MAPK inhibitors. Interestingly, SB203580, a p38 inhibitor, completely inhibited IL-12 expression (Figure 3E). As expected, Cb-EE also suppressed the phosphorylation of p38 observed at 15 and 30 min (Figure 3F). However, the phosphorylation of ERK and JNK was not observed during Cb-EE treatment (data not shown), implying that p38 could be a pharmacological target of Cb-EE.

IL-12 is a cytokine produced by macrophages and dendritic cells that is seen to enhance Th1 response such as IFN-γ production, whether inhibition of IL-12 production by Cb-EE is linked to the suppression of IFN-γ production was examined using PHA- and LPS-treated splenocytes. Although we cannot precisely explain whether stimulation of PHA or LPS exactly mimicked Th1 polarization, higher IFN-γ production conditions with the levels of 10–60 ng/mL was well observed (Figure 4B), assuming that Th1 response may be generated by these stimuli. Interestingly, Cb-EE significantly suppressed the production of IFN-γ from the conditions up to 50% at 100 μg/mL (Figure 4B), exhibiting no cytotoxicity (Figure 4A). No inhibition of IFN-γ promoter activity by Cb-EE was obtained (Figure 4C), when IFN-γ promoter-Luc was transfected to HEK293 cells under the stimulation of PMA and ionomycin A, presumably suggesting that Cb-EE might be ineffective in suppressing the activation of transcription factors to bind to IFN-γ promoter. Otherwise, this result appears to indicate that Cb-EE might indirectly block the. So far, inhibitory mechanism behind IFN-γ production blockage is not yet clears. However, the facts that Cb-EE blocked the expression of IL-12 from LPS-activated macrophages and IFN-γ production was not directly suppressed by Cb-EE strongly imply that this extract is capable of modulating IFN-γ production by controlling the transcriptional activation of IL-12 through targeting the modulation of p38 activation.

The exact components of this plant extract, that could be considered as active principle with inhibitory property against p38/AP-1 activation pathway is not fully understood. None of the reports explaining the phytochemical analysis and biological activities of *Cordyceps bassiana* have been published till date. Because cordycepin, a major active compound isolated from *Cordyceps* spp., is not identified, following high performance liquid chromatography analysis (data not shown), other constituents could be attributed for this activity. In particular, this species contains yellowish colored compounds found in anti-inflammatory subfraction of Cb-EE (data not shown). Therefore, whether these colored compounds are major principle to have IL-12 inhibitory effects will henceforth be continuously tested.

In conclusion, we found that 70% ethanol extract from *Cordyceps bassiana* is able to suppress the expression of IL-12, a cytokine regulating Th1 response, at the transcriptional levels. The inhibitory effect of Cb-EE seemed to be due to AP-1 translocation inhibition, according to immunoblotting analysis done with nuclear fraction and luciferase assay. Cb-EE strongly suppressed the phosphorylation of p38, a prime signal involved in AP-1 translocation. Furthermore, this extract also suppressed IFN-γ production, a representative response of Th1 polarization, in PHA-activated splenocytes, without altering IFN-γ promoter activity. Therefore, our results suggest that Cb-EE can be applied as a Th1 response regulatory herbal medicine. Further in vivo efficacy test

demonstrating its potential therapeutics against Th1 mediated immunological diseases such as chronic atopic dermatitis model will be continued in the next project.

Declaration of interest

This research was supported by Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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